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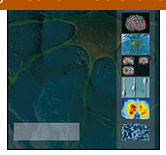

Involvement of serotoninergic and adrenergic systems on the antidepressant-like effect of *E. uniflora* L. leaves essential oil and further analysis of its antioxidant activity

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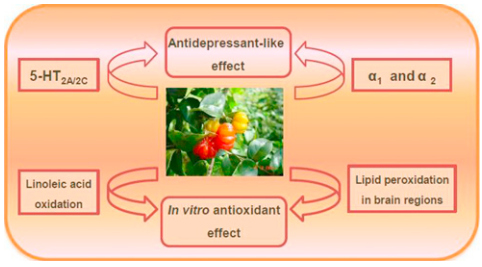
HIGHLIGHTS

- Germacrene B and seline-1,3,7-trien-8-one oxide are the major compounds in essential oil (EO).
- *Eugenia uniflora* leaf EO presented antidepressant-like activity.
- The EO reduced the linoleic acid oxidation.
- The EO reduced the lipid peroxidation on cortex, hippocampus and cerebellum.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work we evaluated antidepressant-like effect of *E. uniflora* leaves EO employing the tail suspension test. The involvement of serotonergic and adrenergic systems was appraised. EO was administered by oral route (p.o.) in mice and the doses of 10 and 50 mg/kg exhibited antidepressant-like action in the TST. The effect of EO (10 mg/kg) was prevented by the pretreatment of mice with ketanserin (5 mg/kg, intraperitoneal), prazosin (0.1 mg/kg, i.p.) and yohimbine (0.1 mg/kg, i.p.). In addition, further analysis of the *in vitro* antioxidant effect of the EO was made against lipid oxidation. The results revealed that EO has a potent antioxidant activity and therapeutic potential for the development of phytomedicines with antidepressant and antioxidant properties.

1. Introduction

Depression is characterized by a wide range of debilitating emotional and physical symptoms such as changed mood and lack of interest in the surroundings [2]. Numerous neural pathways are involved in the pathophysiology of depression, and a great number of neurotransmitters participate in the primary mechanisms of drugs action [5]. Depression has been estimated to affect up to 21% of the world's population and according to WHO's prediction, it will be the second most common disease in 2020 [31].

Some authors have suggested the involvement of oxidative stress in neurological and psychiatric disorders [15]. The oxidative stress occurs when redox homeostasis is challenged by free radicals and reactive oxygen species (ROS), due to either overproductions or deficiencies in antioxidants defenses. The brain is particularly vulnerable to oxidative damage, since it has comparatively high oxygen consumption, modest antioxidant defenses, and a lipid-rich constitution, with the presence of redox-catalytic metals such iron and cupper, and neurotransmitters with reducing potential [18]. The vulnerability of the brain and the association between neurodegenerative changes and psychiatric disorders suggest that oxidative damage mechanisms may be implicated in the

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pathogenesis of depression, and that antioxidant supplementation may be a target in this treatment [6].

In the search for new molecules useful for the treatment of neurological disorders, medicinal plants have emerged as a source for the development of drugs, demonstrating pharmacological potential and playing an important role for patients who respond poorly to conventional treatments [9].

In fact, many studies have addressed the potential of plant-derived essential oils (EOs) on the central nervous system, as natural antidepressants, to relieve anxiety, and stress [4,17,25]. EOs are natural products generally obtained from medicinal plants that exhibit a variety of pharmacological properties [3].

The leaves of *Eugenia uniflora*, popularly known as "pitanga", are used in Brazilian folk medicine in the treatment of rheumatism, stomach diseases, disorders of the digestive tract, hypertension, and also to diminish blood pressure [1,30]. Recently, Colla et al. [12] studied the antidepressant-like effect of a hydro-alcoholic extract of *E. uniflora* leaves in the tail suspension test (TST). However, the extract did not present antidepressant-like effect in the TST even at concentration of 100 mg/kg.

Additionally, we have recently reported the antioxidant, antibacterial and antifungal activities of the pitanga leaf EO and no acute toxicity was observed, with a LD_{50} higher than 200 mg/kg [28]. Based on these promising results and aiming to extend our studies about *E. uniflora* leaves EO, we decide to evaluate its antidepressant-like effect and the possible involvement of serotoninergic and adrenergic systems; moreover, the *in vitro* antioxidant effect of the *E. uniflora* leaves EO was studied against lipid oxidation.

2. Materials and methods

Male Swiss mice of 2–3 months old (25–30 g) were maintaining at 22–25 °C, under 12 h:12 h light/dark cycles, with free access to food and water and at a relative humidity of 60 ± 20%. This study was approved by the Federal University of Pelotas Committee of Animal Experimentation (CEEA 2622) and the experimental protocol was in compliance with National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. All experiments were performed with separate groups of animals (n = 5–6), totalizing 60 animals, approximately, and each animal was used only once.

The *E. uniflora* leaves EO was obtained by steam distillation and its chemical composition was analyzed by gas chromatography coupled with mass spectrometer (GC/MS).

For the *in vitro* assays, EO was dissolved in dimethyl sulfoxide (DMSO) and the control was DMSO alone; for the *in vivo* assays, EO was diluted in canola oil and administered in a constant volume of 10 ml/kg body weight and canola oil alone was the control. The drugs (positive control and antagonists) were dissolved in saline and control animals received appropriate vehicle (saline solution or canola oil).

For the evaluation of the antidepressant-like effect, the animals were pre-treated with different doses of EO (1–50 mg/kg, per oral, (p.o.) or fluoxetine (32 mg/kg, p.o.) or canola oil (10 ml/kg) 60 min before of the tail suspension test (TST). In the TST, animals are placed in an inescapable situation and the antidepressant-like activity is expressed by the decrease of immobility time, an effect that is exhibited by conventional antidepressants [13]. This is a well characterized behavioral model predictive of antidepressant activity.

The total immobility time induced by tail suspension was measured according to the method described by Steru [26]. The mice were suspended on the edge of a table 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time, defined as the absence of escape-oriented behavior, was scored over 6 min as previously described [26].

In order to investigate the possible involvement of serotoninergic system on the antidepressant-like effect of the EO on the TST in mice, distinct groups of animals were pretreated with different antagonists. Animals were pretreated with WAY-100635, a selective antagonist of 5-HT_{1A} receptors (0.1 mg/kg, subcutaneous, s.c.), ketanserin, a non-selective antagonist of 5-$HT_{2A/2C}$ receptors (5 mg/kg, intraperitoneal, i.p) or ondansetron, a selective antagonist of 5-HT_3 receptors (1 mg/kg, i.p.), 15 min before the administration of the EO (10 mg/kg, p.o.) or vehicle (canola oil).

To address the possible involvement of the adrenergic system in the antidepressant-like effect of EO, animals were pretreated with prazosin (1 mg/kg, i.p.), a selective antagonist of α_1 adrenergic receptor, yohimbine (1 mg/kg, i.p.), a selective antagonist of α_2 adrenergic receptor or vehicle and after 15 min they received EO (10 mg/Kg, p.o.) or vehicle and were tested in the TST 1 h later.

The locomotor and exploratory behavior was assessed in the open field test (OFT). Each animal was placed individually at the center of the apparatus and observed for 5 min to record the locomotor (number of segments crossed with the four paws) and exploratory activities (expressed by the number of time rearing on the hind limbs) [29]. The animals were pretreated with *E. uniflora* EO (1–50 mg/Kg, p.o.) and, after 1 h, they were observed in the OFT.

Considering the role of oxidative stress on pathophysiology of depression and to extend our studies about the *in vitro* antioxidant effect of the *E. uniflora* leaves EO, we evaluated the effect of the EO on linoleic acid peroxidation assay and SNP-induced lipid peroxidation on hippocampus, cortex and cerebellum of mice. These brain structures were used since they are implicated in the pathogenesis of depression (cortex and hippocampus) [24] while cerebellum has been indicated as an area under negative functional connectivity from the hippocampus seen in depressive subjects [8].

Linoleic acid was used as a lipid matrix to evaluate the effect of EO on Fe^{2+}-ascorbic acid- induced lipid peroxidation. The ability of EO (10–500 μg/ml) to inhibit the linoleic acid peroxidation was determined at 532 nm by the method of Choi [11].

To evaluate the effect of *E. uniflora* EO in the lipid peroxidation on the hippocampus, cortex and cerebellum, the levels of malondialdehyde (MDA), an end product of lipid peroxidation, were used as a biomarker. For this purpose, mice were euthanized by cervical dislocation and cerebral tissue was rapidly removed, and the hippocampus, cortex and cerebellum were separated. After that, the tissues were immediately homogenized, centrifuged, the pellet was discarded, and a low-speed supernatant (S_1) was used to determine the effects of different concentrations of EO on SNP- induced lipid peroxidation.

SNP was used as a classical inductor of lipid peroxidation [23] and the levels of MDA were determined spectrophotometrically by the thiobarbituric acid reactive substances assay (TBARS), according to the protocol of Ohkawa [21].

The results are presented as means ± standard error mean (SEM). Statistical analysis was performed using a one-way or two-way analysis of variance (ANOVA) followed by the appropriate multiple comparison tests. The *in vitro* assays was performed in duplicate and repeated at least three times; for the *in vivo* assays, six animals were used per group. Differences were considered statistically significant at a p value of 0.05. The IC_{50} (concentration of sample required to inhibit 50% of lipid peroxidation) and ID_{50} (dose necessary to reduce in 50% the immobility time when compared to the control group) values were calculated from the graph of % inhibition *vs.* concentration.

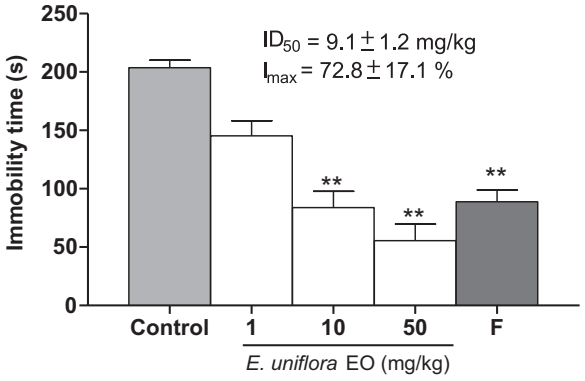

Fig. 1. Effect of acute administration of *E. uniflora* EO on immobility time in the TST. *F* is the positive control fluoxetine (32 mg/kg, p.o.). Values are presented as mean ± standard error mean (SEM) ($n = 6$). Three asterisks represent $p < 0.001$ when compared to the control group by one-way ANOVA followed by the Newman-Keuls multiple range test.

3. Results

The chemical composition of the EO was analyzed by GC/MS. The chromatogram of the EO permitted the identification of seven compounds that comprised approximately 90% of all constituents. The EO contains mainly no-oxygenated sesquiterpenes, followed by oxygenated ones. The major compounds in the EO are germacrene B (22%), selina-1,3,7-trien-8-one-oxide (19%), β-caryophyllene (13%), germacrene A (11.6%), germacrene D (11.4%), selina-1,3,7-trien-8-one (9.5%) and curzerene (4%).

The EO reduced significantly the immobility time at doses equal and higher than 10 mg/kg (58.9% decrease), while fluoxetine reduced by 56.3% (Fig. 1) when compared to the control group (canola oil). The ID_{50} was 9.1 ± 1.2 mg/ml and the maximum inhibition (I_{max}) for the EO on the TST was 72.8 ± 17.1%.

Pretreatment of mice with ketanserin prevents the anti-immobility action of the EO. A two-way ANOVA revealed a significant interaction between the pretreatment and the treatment ($F(1,10) = 8.66$, $p = 0.0147$), as can be seen on Fig. 2. On the other hand, the pretreatment of mice with WAY100635 (($F(1,14) = 2.53$, $p = 0.133$) and ondansetron ($F(1,18) = 0.01$, $p = 0.907$) did not block the antidepressant-like effect of *E. uniflora* leaves EO (Fig. 2A and C).

Table 1
Effect of *E. uniflora* leaves essential oil on linoleic acid peroxidation.

EO (μg/ml)	Inhibition (%)
0[a]	0
10	3.40 ± 3.05
50	27.23 ± 13.74*
100	36.00 ± 12.30*
500	60.67 ± 6.11**
IC_{50} (μg/ml)	325.0 ± 66.1
I_{max} (%)	60.5 ± 6.1

[a] Control (DMSO alone). Values are presented as mean ± SEM. Asterisks represent significant difference of the induced.
* $p < 0.05$.
** $p < 0.01$.
The data were analyzed by one-way ANOVA followed by the Newman–Keuls multiple comparison test.

The pretreatment with adrenergic antagonists revealed that both blocked the antidepressant-like effect of EO, increasing the immobility time when compared to the *E. uniflora* EO group (Fig. 3). Two-way ANOVA revealed a significant interaction between the pretreatment with prazosin and the treatment with EO ($F(1,15) = 7.11$, $p = 0.0176$) and between yohimbine and EO treatments ($F(1,13) = 12.84$, $p = 0.003$).

Considering that the antidepressant-like activity of the *E. uniflora* leaves EO on the TST may be subjected to the presence of components that induce hyperactivity or cause a locomotor problem, given a false positive or negative effect respectively, mice were submitted to the OFT. The administration of EO did not alter significantly the locomotor and exploratory parameters when compared to the control group (data not shown).

The effect of *E. uniflora* EO on the linoleic oxidation is depicted on Table 1. The EO inhibited the oxidation of linoleic acid in concentrations equal and higher than 50 μg/ml with an IC_{50} value of 325.0 ± 66.1 μg/ml and an I_{max} of 60.5 ± 6.1%, while DMSO alone had not effect.

The SNP induced lipid peroxidation in hippocampus, cortex and cerebellum while the *E. uniflora* EO reduced SNP-induced lipid peroxidation at concentrations equal or greater than 50 μg/ml in hippocampus (I_{max} 37.5 ± 12.50%) and 10 μg/ml in cortex (IC_{50}

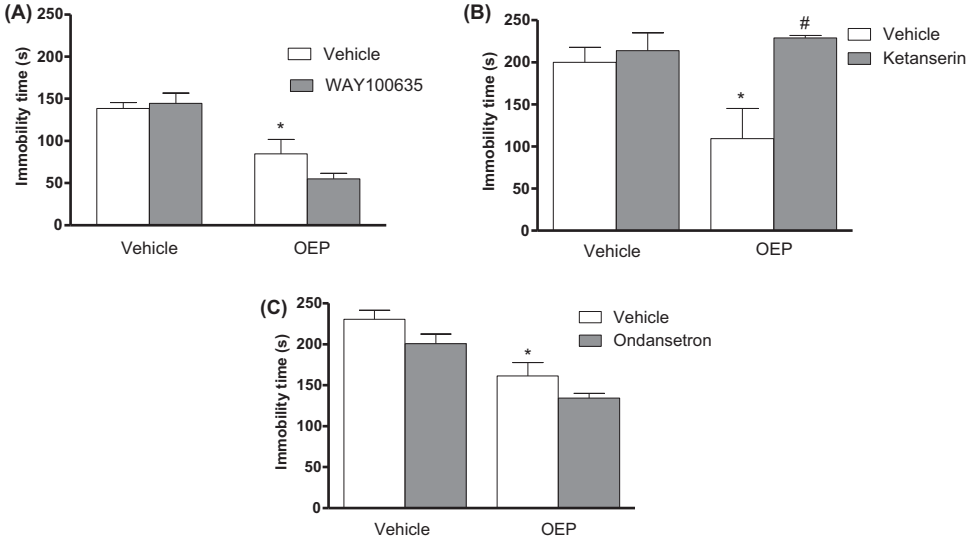

Fig. 2. Effect of pretreatment of mice with (A) WAY100635, (B) ketanserin and (C) ondansetron on the antidepressant-like effect of the *E. uniflora* leaves EO. Values are presented as mean ± standard error mean (SEM) ($n = 6$). One asterisk represents $p < 0.05$ when compared to the vehicle group and (#) represents $p < 0.01$ when compared with EO group by two-way ANOVA followed by Newman-Keuls multiple range test.

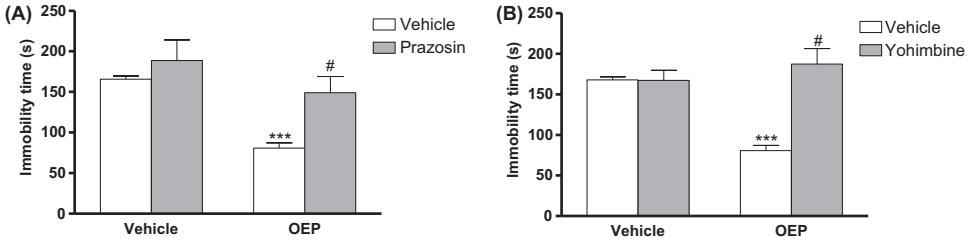

Fig. 3. Effect of pretreatment of mice with (A) prazosin and (B) yohimbine on the antidepressant-like effect of the *E. uniflora* leaves EO. Values are presented as mean ± standard error mean (SEM) ($n = 6$). Three asterisks represents $p < 0.001$ when compared to the vehicle group and (#) represents $p < 0.05$ when compared with EO group by two-way ANOVA followed by the Newman-Keuls multiple range test.

93.30 ± 5.10 μg/ml, I_{max} 53.0 ± 1.90%) and cerebellum of mice (47.0 ± 2.80 μg/ml, I_{max} 53.0 ± 14.6%, Table 2).

For the first time the antidepressant-like effect of *E. uniflora* EO on the TST was reported. This effect is mediated by the modulation of serotoninergic and adrenergic systems. Interestingly, *in vitro* assays revealed that the EO inhibits the lipid oxidation in the assays of linoleic acid oxidation and SNP-induced lipid peroxidation in cerebral structures.

4. Discussion

In the present work, we demonstrate that *E. uniflora* EO produced antidepressant-like effect in the TST at doses of 10 and 50 mg/kg. Considering the negative results of Colla et al. [12] regarding the antidepressant-like effect of hydro-alcoholic extract of *E. uniflora* L leaves in the TST, the hydro alcoholic extract did not present antidepressant-like effect in the TST, probably, the amount of volatile compounds present in the EO, like oxygenated and non-oxygenated sesquiterpenes are responsible for its antidepressant-like effect.

Many studies have suggested that the activation of 5-HT_2 receptors can be related in the regulation of mood disorders [10]. These receptors are widely distributed throughout brain structures that are important on the pathophysiology of depression, like pre-frontal cortex and hippocampus [19]. Conventional antidepressants are based on the enhancement of monoamines in the brain, like serotonin, norepinephrine and dopamine that are involved in the pathophysiology of depression [14].

According with our results, the antidepressant-like effect of *E. uniflora* leaves EO involves the modulation of receptors 5-$HT_{2A/2C}$, since the pretreatment of mice with ketanserin, an antagonist of these receptors, significantly prevent the anti-immobility effect of the EO.

Besides the serotoninergic, the adrenergic system is classically implicated in the pathophysiology of depression [16], and compounds that affect the adrenergic neurotransmission, such as noradrenaline reuptake inhibitors or monoamine oxidase inhibitors, are currently used to treat depression [22].

In this study, the pretreatment of mice with prazosin, a selective antagonist of α_1 adrenoreceptor, has blocked the antidepressant-like effect of *E. uniflora* EO. Furthermore, the pretreatment of animals with yohimbine, a α_2 adrenoreceptor antagonist, improved the immobility time. This finding indicates that α_2 is also involved in the action of EO.

Thus, the antidepressant-like effect of the *E. uniflora* EO appears to be involved, at least in part, with serotoninergic and adrenergic systems.

Recent clinical evidences have showed the co-existence between increased oxidative stress and depression symptoms in patients [20]. Since the *E. uniflora* EO presented antidepressant-like effect, we extend our studies to evaluate its *in vitro* antioxidant effect.

E. uniflora EO decreased the levels of linoleic acid oxidation in concentrations ranging from 50 to 500 μg/ml and presents effect against the SNP-induced lipid oxidation on the studied cerebral regions. Reactive oxygen derived species (ROS) partially reduced are produced as part of normal physiological and metabolic processes in aerobic organisms. Excessive production of ROS can lead to oxidative stress, an imbalance between the pro-oxidant and antioxidant species [18]. Oxidative stress is known to affect lipid membrane through lipid peroxidation and also to oxidize proteins and DNA.

Alterations in phospholipids may induce changes in membrane viscosity and in various neurotransmitter systems like serotonin (5-HT) and noradrenaline [27]. However, it is not clear if oxidative stress is a cause or a result of depression [7].

Table 2
Effect of *E. uniflora* L leaves EO on SNP-induced lipid peroxidation.

	SNP-induced lipid peroxidation (nmol MDA/g tissue)		
	Hippocampus	Cortex	Cerebellum
Control	769.2 ± 23.83*	628.90 ± 150.40*	632.90 ± 45.24*
Induced	1400 ± 163.2#	1458.00 ± 167.20#	1220.0 ± 77.91#
EO 1 μg/ml	1744.0 ± 268.1	1793.0 ± 236.50	1437.0 ± 280.50
EO 10 μg/ml	888.90 ± 14.14	568.80 ± 195.00**	758.10 ± 109.50**
EO 50 μg/ml	775.10 ± 176.80*	797.30 ± 163.90*	586.10 ± 40.87**
EO 100 μg/ml	647.80 ± 228.40*	791.90 ± 86.10*	515.70 ± 125.00**
IC_{50} (μg/ml)	–	93.30 ± 5.10	47.0 ± 2.80
I_{max} (%)	37.5 ± 12.5	53.0 ± 1.90	53.0 ± 14.60

Values are presented as mean ± SEM. Asterisks represent significant difference of the induced (tissue + DMSO + SNP).

* $p < 0.05$.

** $p < 0.01$.

\# Represent significant difference from the control (tissue alone, $p < 0.05$).

The data were analyzed by one-way ANOVA followed by the Newman–Keuls multiple range test.

5. Conclusion

The present work demonstrated that the antioxidant activity and the antidepressant-like effect of *E. uniflora* leaves EO are mediated by the modulation of serotoninergic and adrenergic systems. Together, these results suggest that the *E. uniflora* EO may have therapeutic value. However, more studies are necessary to elucidate others possible mechanisms involved on the antidepressant-like effect of the EO as well as to evaluate its effect in a long term exposition.

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